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Analysis of active-site amino-acid residues of human serum paraoxonase using competitive substrates

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Serum paraoxonase (PON1) is a calcium-dependent six-fold β-propeller protein structurally similar to the di-isopropylfluorophosphatase (DFPase) found in the squid *Loligo vulgaris*. Human serum paraoxonase (HuPON1) has been shown to hydrolyze an array of substrates even though relatively little is known about its physiological role(s) or its catalytic mechanism. Through site-directed mutagenesis studies, designed from a DFPase-like homology model, and from a crystal structure of a hybrid PON1 molecule, amino-acid residues essential for enzyme function, including H115 and F222, have been identified. It was shown previously that, when H115 is replaced with tryptophan, the resulting enzyme hydrolyzes paraoxon but not phenyl acetate. This study shows that, when present simultaneously, phenyl acetate competitively inhibits paraoxon hydrolysis by H115W. Conversely, when F222 is replaced with tyrosine, mutant F222Y can hydrolyze phenyl acetate but not paraoxon. The presence of DFP, an inhibitor of both arylesterase and paraoxonase activities of wild-type HuPON1 (mean $K_i = 0.48 \pm 0.15$ mM), has no effect on the ability of F222Y to catalyze the hydrolysis of phenyl acetate, suggesting that the F222Y mutant is unable to bind DFP. Together, the results suggest that, in wild-type HuPON1, H115 and F222 are important in determining substrate binding and specificity, but are not likely to be directly involved in substrate hydrolysis.

Human serum paraoxonase (HuPON1; EC 3.1.8.1) is a 43–45 kDa glycosylated protein comprising 354 amino acids [1]. It is synthesized in the liver and secreted into the bloodstream where it is found bound to high-density lipoprotein in the circulation [2–4]. The enzyme hydrolyzes multiple classes of substrates [1,4–10]. Among these classes are aryl esters (including phenyl acetate), lactones, and organophosphorus compounds including paraoxon and the chemical warfare agents sarin, soman, and VX [6,11–13]. Although its catalytic efficiency against these compounds is low, it is the capacity to hydrolyze organophosphates *in vivo* that makes HuPON1 an extremely attractive bioscavenger candidate [14,15] against organophosphorus intoxication.

It may be possible to produce site-directed variants of HuPON1 to create enzymes with enhanced stability and catalytic activity against organophosphorus compounds [16,17], increasing their efficacy as anti-organophosphorus bioscavengers. Although the recently published di-isopropylfluorophosphatase-like HuPON1 homology model (Protein Database accession code 1XHR) [15,18] and gene-shuffled PON1 variant crystal structure (Protein Database accession code 1V04) [19] have provided invaluable insight into the overall structure of the enzyme, the exact catalytic mechanism remains unclear. For example, Harel *et al.* [19] suggest that the histidine dyad H115-H134 is directly involved in the catalytic mechanism of PON1. However, a H115W substitution

Abbreviations
DFP, di-isopropylfluorophosphate; HuPON1, human serum paraoxonase; PON1, serum paraoxonase.

mutant retained catalytic activity against paraoxon [15], suggesting that H115 is not a critical catalytic residue for organophosphorus hydrolysis.

To provide additional insight into the functional components of the active site of HuPON1, the organophosphorus compound di-isopropylfluorophosphate (DFP) was used as an inhibitor of both arylesterase and paraoxonase activities of the wild-type enzyme [1]. DFP, paraoxon, and phenyl acetate were used in various combinations with active-site mutants (F222Y, H115W, and H115W/N133S) previously reported to have enzymatic activity against either phenyl acetate or paraoxon but not both [15]. The aim of this study was to determine whether these mutations affected the capacity of the enzyme to bind to the nonhydrolyzed substrate(s).

Results

Effect of DFP on the enzymatic properties of HuPON1

In the absence of DFP, the mean K_M of wild-type HuPON1 for phenyl acetate at pH 8.0 was 0.61 ± 0.09 mM (Table 1), and at pH 10.5 it was 1.09 ± 0.45 mM (data not shown). Relative V_{max} values remained constant in the presence of 0.65 and 1.30 mM DFP, whereas the K_M values increased. The inhibition constant, K_i, of DFP for phenyl acetate hydrolysis at pH 8.0 was determined to be 0.52 ± 0.16 mM. Wild-type HuPON1 bound paraoxon with a K_M of 0.31 ± 0.06 mM at pH 8.5. Comparable to phenyl acetate, K_M increased whereas relative V_{max} did not change in the presence of either 0.50 or 2.50 mM DFP. The K_i of DFP for paraoxonase was determined to be 0.44 ± 0.13 mM.

Phenyl acetate inhibits paraoxonase activity of HuPON1

Kinetic parameters were also determined for paraoxonase using phenyl acetate as a competitive substrate (assay performed in paraoxonase buffer, pH 8.5; see Experimental procedures). The uninhibited K_M for paraoxon was determined to be 0.31 ± 0.06 mM at pH 8.5 and 0.25 ± 0.08 mM at pH 10.5 (Table 1). Akin to the effects observed with the addition of DFP, the addition of phenyl acetate caused the relative K_M values for paraoxon to increase whereas the derived V_{max} remained constant (data not shown). At pH 8.5 and 10.5, phenyl acetate yielded K_i values of 0.47 ± 0.17 mM and 0.91 ± 0.19 mM, respectively (Table 1).

Table 1. K_i values for inhibition of the catalytic activity of recombinant HuPON1 by DFP or phenyl acetate. Arylesterase assays were performed in 50 mm Tris/HCl/1 mm $CaCl_2$, pH 8.0, and paraoxonase activities were assayed in 50 mm glycine/NaOH/1 mm $CaCl_2$, at pH 8.5 or pH 10.5, as indicated. Kinetic parameters are mean ± SD from at least three independent experiments.

pH	Substrate	K_M (mM)	Inhibitor	K_i (mM)
8.0	Phenyl acetate	0.61 ± 0.09	DFP	0.52 ± 0.16
8.5	Paraoxon	0.31 ± 0.06	DFP	0.44 ± 0.13
8.5	Paraoxon	0.31 ± 0.06	Phenyl acetate	0.47 ± 0.17
10.5	Paraoxon	0.25 ± 0.08	Phenyl acetate	0.91 ± 0.19

DFP does not inhibit arylesterase activity in mutant F222Y

In the absence of DFP, the mutant F222Y exhibits a K_M for phenyl acetate of 0.81 ± 0.48 mM (Table 2). Inhibition assays were carried out by adding 1.30 mM DFP under the same conditions as those for wild-type HuPON1. In the presence of DFP, the apparent K_M value for phenyl acetate was 0.90 ± 0.49 mM (data not shown), suggesting that DFP does not bind F222Y. Relative V_{max} values for phenyl acetate in the presence or absence of DFP were consistent. Mutants H115W and H115W/N133S respond differently to phenyl acetate as an inhibitor of paraoxonase activity. To compare results with previous findings [15], paraoxonase activities of mutants H115W and H115W/N133S were assayed in paraoxonase buffer at pH 10.5. Consistent with published results [15], the K_M for paraoxon of the single mutant, H115W, was 0.43 ± 0.07 mM and that of the double mutant, H115W/N133S, was 0.19 ± 0.05 mM. Inhibition assays were carried out under the same conditions with the addition of 1.50 mM phenyl acetate. The presence of phenyl acetate increased the apparent K_M of H115W for

Table 2. Kinetic parameters of recombinant HuPON1 mutants for the hydrolysis of phenyl acetate and paraoxon in the presence of competitive inhibitors. Paraoxonase activities were assayed in 50 mM glycine/NaOH/1 mM $CaCl_2$, at pH 10.5, and arylesterase activities were assayed in 50 mM Tris/HCl/1 mM $CaCl_2$, pH 8.0. Kinetic parameters are mean ± SD from at least three independent experiments. nd, Inhibition not detectable at 1.50 mM phenyl acetate for H115W/N133S and 1.30 mM DFP for F222Y, respectively.

Mutants	Substrate	K_M (mM)	Inhibitor	K_i (mM)
H115W[a]	Paraoxon	0.43 ± 0.07	Phenyl acetate	1.95 ± 0.91
H115W/N133S[a]	Paraoxon	0.19 ± 0.05	Phenyl acetate	nd
F222Y[b]	Phenyl acetate	0.81 ± 0.48	DFP	nd

[a] Phenyl acetate or [b] paraoxon hydrolysis was not detectable [15].

paraoxon. The K_M of the double mutant, H115W/N133S, for paraoxon was not affected by the presence of phenyl acetate (Table 2). The K_i value of phenyl acetate for the H115W mutant was found to be at least twice as high (1.95 ± 0.91 mM) as that of the wild-type HuPON1 (0.91 ± 0.19 mM) at pH 10.5.

Discussion

DFP as an inhibitor of substrate catalysis by HuPON1

The active site of PON1 is characterized by a single catalytic calcium ion [12,20–22]. Removing this ion inactivates the enzyme's ability to hydrolyze both phenyl acetate and paraoxon [23], suggesting that the two esters share the same active site. If true, a competitive substrate will inhibit both phenyl acetate and paraoxon hydrolysis. The organophosphorus compound DFP, another substrate of PON1, was screened for its potential to act as a competitive inhibitor. The kinetic parameter K_M for both paraoxonase and arylesterase activities of wild-type HuPON1 increased in the presence of DFP (data not shown). The calculated V_{max} (y intercept of Lineweaver–Burk plot) did not deviate greatly from that of the uninhibited wild-type for either paraoxon or phenyl acetate hydrolysis (data not shown). These results suggest that the organophosphorus compound DFP competitively inhibits paraoxonase and arylesterase activities of HuPON1, and further that DFP, phenyl acetate and paraoxon occupy the same active site. Phenyl acetate was also found to be capable of competitively inhibiting the paraoxonase activity of recombinant HuPON1 (Table 1). This result is in contrast with those of Gan *et al.* [1], who reported mixed-type inhibition of paraoxonase by phenyl acetate using PON1 purified from human serum. Nonetheless, the K_M and K_i values reported by Gan *et al.* are in close agreement with those reported here. Phenyl acetate hydrolysis was not monitored in the presence of paraoxon as an inhibitor because phenol (the product of phenyl acetate catalysis) and paraoxon share an absorbance peak at A_{270}.

pH Dependence of the affinity of HuPON1 for phenyl acetate

To probe the pH dependence of phenyl acetate inhibition of paraoxonase activity, paraoxon hydrolysis was monitored at two different pH values in the absence or presence of phenyl acetate. The wild-type HuPON1 K_M for paraoxon increased upon addition of phenyl acetate at both pH 8.5 and 10.5. Interestingly, the K_i of phenyl acetate at pH 8.5 was only half that of the K_i at pH 10.5. Given that the uninhibited wild-type K_M for paraoxon at pH 8.5 and at 10.5 were very similar (Table 1), and the K_M values for phenyl acetate at pH 8.0 and 10.5 differed (0.61 ± 0.09 mM and 1.09 ± 0.45 mM, respectively), the data support the idea that an amino-acid residue at or near the active site deprotonates between pH 8.5 and 10.5, decreasing affinity for phenyl acetate but not for paraoxon. Candidate amino-acid residues with pKa values in an appropriate range include cysteine, tyrosine, and lysine. Whereas multiple lysine and tyrosine residues (e.g. K70, K81, K192, Y190, and Y294) are located near the proposed active site of wild-type HuPON1, cysteine 284 (C284) is of particular interest because mutations at this residue have been shown to alter both arylesterase and paraoxonase activity [15,22].

Active-site residues involved in paraoxonase and arylesterase activities

Residue F222 is postulated to influence substrate binding [15,18]. Replacing phenylalanine at residue 222 with aspartate (D) produced a mutant (F222D) with no detectable enzymatic activity, whereas the conservatively substituted F222Y lost paraoxonase but retained arylesterase activity, with a 1.5-fold increase in K_M for phenyl acetate [15]. The presence of 1.30 mM DFP did not substantially alter the apparent K_M for phenyl acetate hydrolysis, implying that the substitution of Y for F at position 222 eliminates DFP binding. Consequently, the findings reaffirm our previous hypothesis identifying F222 as a residue important for conferring substrate specificity [15].

On the basis of the crystal structure of a hybrid PON1 enzyme, Harel *et al.* [19] hypothesized that residues H115 and H134 form a catalytic dyad responsible for the enzymatic activities of PON1. Substitution of either of these residues should therefore eliminate all enzymatic activity. As previously shown, substituting either tyrosine (Y) or tryptophan (W) at residue H134 eliminates both arylesterase and paraoxonase activities, but substituting W at H115 ablates phenyl acetate hydrolysis while only moderately decreasing paraoxonase affinity [15]. Interestingly, an unintended double mutant with a histidine to tryptophan mutation at residue 115 and a asparagine to serine at residue 133 (H115W/N133S) also could not hydrolyze phenyl acetate, but exhibited wild-type K_M for paraoxon [15]. To further address the question of whether H115 actually plays a

mechanistic role in catalysis, we examined whether the mutants H115W and H115W/N133S lacked the ability to bind or hydrolyze phenyl acetate. Phenyl acetate did not inhibit paraoxon hydrolysis in the double mutant H115W/N133S, suggesting that it is not binding to the active site of this mutant. In contrast, when phenyl acetate was used as an inhibitor of paraoxon hydrolysis by H115W, competitive inhibition was observed, indicating that H115W is capable of binding phenyl acetate, but is unable to hydrolyze this substrate. By extension, it is likely that both residues N133 and H115 play important roles in determining PON1 substrate specificity, but not catalysis, of paraoxon. This conclusion is contrary to a previous report by Harel *et al.* [19], but is consistent with a more recent report from the same group [24]. It remains possible that, despite substantial evidence that phenyl acetate and paraoxon target the same active site in PON1 (presented herein and in [1,25]), the amino-acid residues responsible for electron transfer during the enzymatic hydrolysis of these substrates may differ [25].

Experimental procedures

Production of recombinant HuPON1

Wild-type and mutant PON1 were produced as described [15]. In brief, cDNAs encoding either wild-type or mutated HuPON1 were ligated into the plasmid pcDNA3 (Invitrogen, Carlsbad, CA, USA) and verified by sequencing. PON1-containing plasmids were transiently transfected by calcium phosphate precipitation into human 293T embryonic kidney cells. PON1 expression in cultured supernatants was detected by western blot and confirmed by assaying enzymatic activity.

Enzyme assays

Single-substrate assays were run initially to determine kinetic parameters in the absence of a second substrate or inhibitor. All assays were performed at 25 °C.

Arylesterase activity

Wild-type HuPON1 arylesterase activity was determined using phenyl acetate (Sigma-Aldrich, St Louis, MO, USA) at six concentrations (from 0.26 to 3.3 mM) in 50 mM Tris/HCl/1 mM $CaCl_2$ at pH 8.0 or 10.5, as described [12,15], with the following minor modifications. After the uninhibited enzymatic parameters had been determined, they were determined again in the presence of either 0.65 or 1.30 mM DFP dissolved in methanol (Sigma-Aldrich). Inhibited arylesterase activity of mutant F222Y was only determined at 1.30 mM DFP. Assays were carried out in a total volume of 1.0 mL.

Paraoxonase activity

Paraoxonase activity was determined using 0.26–2.6 mM paraoxon (diethyl *p*-nitrophenyl phosphate; Sigma-Aldrich) in 50 mM glycine/NaOH/1 mM $CaCl_2$ [26–28] at pH 8.5 or pH 10.5, as indicated in the text for wild-type HuPON1. Paraoxonase activity of mutants H115W and H115W/N133S was also determined in the presence of phenyl acetate (at 1.50 mM) at pH 10.5. Kinetic parameters for 'inhibited' wild-type HuPON1 paraoxonase activity were determined at 0.50 and 2.50 mM DFP, and at 0.45 and 1.50 mM phenyl acetate. Assays were carried out in a total volume of 1.0 mL.

Calculation of kinetic constants

Enzymatic parameters of wild-type HuPON1 were derived as previously described [15] by fitting experimental data to the Michaelis–Menten and Lineweaver–Burk equations using GraphPad PRISM software version 3.0 (GraphPad Software, San Diego, CA, USA) and EXCEL 2000. Values for K_M and relative values for V_{max} were derived for supernatants from two or more independent transfections of each PON1 construct. As only small quantities of the mutant enzymes were produced, accurate recombinant PON1 concentrations could not be obtained. Therefore, only apparent K_M values are reported here. Rates of spontaneous and/or albumin-mediated hydrolysis of the substrates in arylesterase and paraoxonase assays were accounted for using supernatants from cells transfected with empty vector; these background rates were subtracted from each sample to yield net rates of hydrolysis.

To determine competitive inhibition constants using GraphPad PRISM software, the formula $K_i = [I]/[(K_{obs.m}/K_M) - 1]$, where [I] is the inhibitor concentration and $K_{obs.m}$ is the observed K_M in the presence of inhibitor, was used. The value of K_i was further refined by plotting $[S]/V_0$ vs. [S] to determine the y intercept (b_{int}). The y intercept value was then used to solve for K_i, where $K_i = [I]/[(b_{int}V_{max})/K_M] - 1$.

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